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Effects of Salt Stress on Amino Acid, Organic Acid, and Carbohydrate Composition of Roots, Bacteroids, and Cytosol of Alfalfa (*Medicago sativa* L.)[1]

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ABSTRACT

Ethanol-soluble organic acid, carbohydrate, and amino acid constituents of alfalfa (*Medicago sativa*) roots and nodules (cytosol and bacteroids) have been identified by gas-liquid chromatography and high performance liquid chromatography. Among organic acids, citrate was the predominant compound in roots and cytosol, with malonate present in the highest concentration in bacteroids. These two organic acids together with malate and succinate accounted for more than 85% of the organic acid pool in nodules and for 97% in roots. The major carbohydrates in roots, nodule cytosol, and bacteroids were (descending order of concentration): sucrose, pinitol, glucose, and ononitol. Maltose and trehalose appeared to be present in very low concentrations. Asparagine, glutamate, alanine, γ-aminobutyrate, and proline were the major amino acids in cytosol and bacteroids. In addition to these solutes, serine and glutamine were well represented in roots. When alfalfa plants were subjected to 0.15 M sodium chloride stress for 2 weeks, total organic acid concentration in nodules and roots were depressed by more than 40%, whereas lactate concentration increased by 11, 27, and 94% in cytosol, roots, and bacteroids, respectively. In bacteroids, lactate became the most abundant organic acid and might contribute partly to the osmotic adjustment. On the other hand, salt stress induced a large increase in the amino acid and carbohydrate pools. Within the amino acids, proline showed the largest increase, 11.3-, 12.8-, and 8.0-fold in roots, cytosol, and bacteroids, respectively. Its accumulation reflected an osmoregulatory mechanism not only in roots but also in nodule tissue. In parallel, asparagine concentration was greatly enhanced; this amide remained the major nitrogen solute and, in bacteroids, played a significant role in osmoregulation. On the contrary, the salt treatment had a very limited effect on the concentration of other amino acids. Among carbohydrates, pinitol concentration was increased significantly, especially in cytosol and bacteroids (5.4- and 3.4-fold, respectively), in which this cyclitol accounted for more than 35% of the total carbohydrate pool; pinitol might contribute to the tolerance to salt stress. However, trehalose concentration remained low in both nodules and roots; its role in osmoregulation appeared unlikely in alfalfa.

The effects of saline soils on plant growth have been a focus of research for nearly 100 years because salt stress is a major stress limiting crop productivity. Salt tolerance of plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes. These aspects have been covered periodically in several reviews (8, 9). Survival and growth in saline environments is the result of adaptative processes such as ion transport and compartmentation, osmotic solute synthesis and accumulation that lead to osmotic adjustment, and protein turnover for cellular repair (14, 16). Although the mechanisms of salt injury and salt tolerance of whole plant have been studied extensively, studies at the cellular, organellar, and molecular levels are still limited (10).

In legumes, salt stress from 50 to 200 mM NaCl also imposes a significant limitation of productivity related to the adverse effects on the growth of the host plant, the root nodule bacteria, symbiotic development, and finally the nitrogen fixation capacity (1, 30). Identification of intracellular solutes and their modification in salt-stress condition could be of potential use in selection of salt-adapted cultivars. In addition, studies of metabolite concentrations in nodules, and especially in bacteroids, are essential to understand the consequence of salt stress on the functioning of legume nodules at the molecular level. However, little is known about organic solute changes induced in legumes subjected to salt stress, and information is available only at the whole plant level. Alfalfa (*Medicago sativa* L.) represents an important leguminous forage crop throughout the world and reduces atmospheric nitrogen in association with the soil bacterium *Rhizobium meliloti*. Although there has been some effort to analyze the concentrations and proportions of soluble nitrogenous compounds in alfalfa nodules (24, 25), the effects of salt stress on solutes in roots and nodules have not been established.

In the present study, identification and quantitative determinations are reported for organic acids, carbohydrates, and amino acids in roots and in bacteroids and cytosol of alfalfa nodules. In addition, we have investigated the changes in these solutes under salt stress and determined whether or not the level of some organic compounds could be used as an indicator of the degree of salt stress encountered by the plants.

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MATERIALS AND METHODS

Plant Growth

Plants were obtained by clonal propagation of shoot cuttings from a single plant of alfalfa, *Medicago sativa* L. (cv Europe), as previously described (6). They were inoculated with *Rhizobium meliloti* 102F34 and grown in aeroponic conditions with a nitrogen-free salts solution (6). Three weeks after inoculation, the plants were subjected to salt stress by adding NaCl to the growth medium (final concentration 0.1 or 0.15 M) or were maintained on NaCl-free medium. Salinization was applied for 2 weeks and nodules were harvested. Two different plant cultures were prepared for each condition. During the whole process, plants were grown in a greenhouse with 16 h photoperiod (1500–1800 $\mu E\ m^{-2}\ s^{-1}$). Day and night temperatures were approximately 27 and 20°C, respectively. Nodules were pulled from roots at 34 d after inoculation and immediately chilled (1°C). Subsamples of 3.0 g of nodules and 10.0 g of nodule-free roots were used for analysis.

Extraction and Preparation of Fractions

Although we were aware of the heterogeneity of the structural components of the nodule, it would have been technically difficult to separate, quickly enough, the cortex, the meristem, and the interior regions from the large quantity of nodules needed for each quantitative determination. Consequently, chilled whole nodule samples were extracted as previously described (21). They were ground in a mortar in 10 mL of deionized water (1°C), and the mixture was filtered through four layers of cheesecloth. The filtrate was immediately centrifuged at a speed of 20,000 rpm for 2 min in a JA-20 angle-head rotor (48,400*g*) in a Beckman J2–21 centrifuge. Solutes from the supernatant (cytosol), which contained most of the mitochondria, were immediately extracted with 20 mL of hot (75°C) 95% (v/v) ethanol. The surface of the bacteroid pellet was rinsed with 1 mL of ice-cold water in order to eliminate the contaminants (cytosol and few sedimented mitochondria); the rinse was discarded. The bacteroid pellet was then extracted with hot ethanol (15 min at 75°C) as previously described (21). Roots were cut with scissors into 1 or 2-cm fragments, and 100 mL of 95% (v/v) ethanol was added. The mixture was ground three times for 2 min at 4°C in a Sorvall Omni Mixer at maximum speed. The resulting mixture was treated at 75°C for 15 min, cooled, and centrifuged (10 min at 10,000*g*). Solids were reextracted twice with 80% (v/v) ethanol. The combined ethanol extracts from bacteroids, cytosol, and roots were dried *in vacuo,* and solids were dissolved in 1.0, 3.0, and 10.0 mL of water, respectively, and stored at 1°C over approximately 20% chloroform. Extracts were divided into neutral, anionic, and cationic fractions on QAE- and SP-Sephadex ion-exchange columns (21). The internal standards for organic acid analysis (tartarate) and amino acid analysis (norleucine) were added to samples prior to ion-exchange fractionation.

Analysis of Metabolites

Analysis of carbohydrates was performed as described earlier (21). An aliquot of the neutral fraction was dried and treated with hydroxylamine to form oxime derivatives (Pierce Chemical Co). Then the mixture was analyzed by GLC on a 2 m × 2 mm (i.d.) packed column containing solid support coated with 3% OV-17. The internal standard was β-phenyl glucose.

Amino acids from the cationic fraction were analyzed by HPLC with the Waters Pico Tag method. It involves the formation of phenylthiocarbamyl derivatives which are analyzed on a 3.9 × 300 mm Pico-Tag free amino acid analysis column (PIN 10 950). The gradient system consisted of two solvents, Pico-Tag 1 and 2, and the gradient program was as described in the Pico-Tag method. The HPLC system consisted of a Waters (Milford, MA) No. 680 automated gradient controller, two No. 510 pumps, No. 712 WISP auto injector, No. 490 detector, No. 740 data module, and a Bio-Rad column heater (21).

Analysis of organic acids involved the formation of phenacyl ester derivatives, which were analyzed by HPLC with a 3.9 × 150 mm Novapak C 18 (Waters) column. The air-dried anionic fraction was dissolved in 2.5 mL of water and titrated to pH 7.0 to 7.5 with 0.3 N KOH and an electronic digital pipet (Rainin Instrument Co., Woburn, MA). The K salts were redried under air and redissolved to give suitable quantities in a 250 μL aliquot (21). Derivatization was carried out in 0.08 M phosphate buffer as described (2), except that the reaction mixture was scaled down to a total volume of 1 or 2 mL and 13 × 100 mm tubes with Teflon-lined screw caps were used (21). Analysis was performed with solvent A, consisting of 35% (v/v) methanol, and solvent B, which was 100% methanol, and a flow rate of 1.0 mL/min. Initial condition was 90% A/10% B, followed by a linear gradient to 30% A/70% B at 26 min, held for 1 min, and returned to the initial condition at 28 min. This condition was maintained for 8 min before the next injection; thus, the total run time was 36 min. This program corresponds to the modified gradient described earlier (21). Injection volume was 15 μL, column temperature was constant at 40°C, and peaks were detected at 245 nm.

Identification of organic acids and amino acids was based on precise co-chromatography of sample and standard peaks under a variety of analytical conditions, and identification of carbohydrates was based on this criterion plus mass spectrometry of trimethylsilyl derivatives (21). Quantitative analysis was based on standard curves established prior to analysis of each set of samples and verified from time to time during sample analysis.

Data are expressed as concentrations of metabolites in bacteroids or cytosol from 1 g fresh weight of nodules and concentrations of metabolites in 1 g fresh weight of roots. To allow conversion of the data obtained with cytosol and bacteroids to a protein basis, a 3-g nodule sample was ground and processed as described above. Cytosol was diluted in water to a known volume, and aliquots were analyzed for protein (13). Bacteroids were also resuspended in a known volume of water, and aliquots were taken for determination of dry weight. Other aliquots were sonicated for 5 min and centrifuged (12,000*g*, 5 min), and the supernatant was used for soluble protein analysis.

RESULTS AND DISCUSSION

Concentration of Organic Acids, Amino Acids, and Carbohydrates in Alfalfa Nodules and Roots

Metabolites found in cytosol, bacteroids, and roots from alfalfa plants grown under unstressed conditions are listed Table I. Data are expressed as nmol of metabolites/g fresh weight of nodules (bacteroids, cytosol) or roots. The relationship to protein content or dry weight was as follows: 1 g fresh weight of nodules contained 14.5 ± 0.5 mg protein in cytosol, 5.6 ± 0.3 mg soluble protein, or 15.3 ± 0.7 mg dry weight in bacteroids, and 1 g fresh weight of roots corresponded to 73.6 ± 3.5 mg dry weight.

During the isolation of bacteroids from the nodules, compounds might have exchanged between the bacteroids and the cytosol. These exchanges represent a potential difficulty for accurate quantitative determinations of metabolite concentrations in both compartments. In previous experiments, in order to estimate these exchanges, the bacteroid/cytosol mixtures from ground soybean nodules were allowed to stand at 1°C for times ranging from 0 to 44 min before analysis of the metabolites (21). Reasonable estimates of concentrations in bacteroids and cytosol were obtained with replicate samples obtained within 10 or 15 min after the nodules were homogenized.

Table I. *Concentration of Metabolites in Cytosol, Bacteroids, and Roots from Alfalfa Plants Grown in a NaCl-Free Medium*

Metabolites	Cytosol	Bacteroids	Roots
	nmol g^{-1} fresh wt of nodules[a]		*nmol g^{-1} fresh wt*[a]
Organic acids			
Citrate	5,228 ± 1,165	157 ± 4	11,372 ± 807
Malate	4,297 ± 1,267	206 ± 3	6,366 ± 102
Malonate	2,348 ± 76	287 ± 40	4,018 ± 13
Succinate	1,546 ± 559	114 ± 6	696 ± 60
Fumarate	550 ± 163	36 ± 6	88 ± 2
Lactate	481 ± 26	70 ± 9	315 ± 72
Benzoate	418 ± 64	24 ± 2	247 ± 2
Total	14,868	894	23,102
Carbohydrates			
Sucrose	15,525 ± 1,551	750 ± 130	11,874 ± 101
D-Pinitol	4,117 ± 45	216 ± 57	2,146 ± 141
Glucose	3,262 ± 499	128 ± 35	768 ± 36
Ononitol	2,436 ± 277	118 ± 20	744 ± 6
D-*chiro*-Inositol	1,473 ± 594	92 ± 52	ND[b]
myo-Inositol	1,095 ± 137	68 ± 22	674 ± 6
Fructose	519 ± 97	27 ± 2	725 ± 138
Maltose	177 ± 42	18 ± 3	64 ± 12
α-α-Trehalose	101 ± 36	8 ± 1	83 ± 5
Total	28,705	1,425	17,078
Amino acids			
Asparagine	6.978 ± 1,483	372 ± 7	293 ± 33
Glutamate	2,571 ± 181	173 ± 28	572 ± 51
Alanine	1,203 ± 16	68 ± 15	142 ± 5
γ-Aminobutyrate	908 ± 83	39 ± 7	405 ± 9
Proline	407 ± 79	22 ± 9	50 ± 10
Aspartate	278 ± 2	2 ± 1	41 ± 8
Valine	243 ± 68	14 ± 6	37 ± 1
Glycine	198 ± 12	14 ± 1	60 ± 3
Leucine	187 ± 68	12 ± 6	34 ± 3
Lysine	178 ± 71	4 ± 2	19 ± 1
Threonine	159 ± 19	8 ± 1	40 ± 8
Isoleucine	152 ± 47	9 ± 4	19 ± 1
Glutamine	144 ± 28	7 ± 1	136 ± 23
Phenylalanine	136 ± 15	9 ± 3	57 ± 7
Arginine	116 ± 26	4 ± 1	32 ± 1
Tryptophane	76 ± 3	5 ± 1	28 ± 1
Tyrosine	64 ± 24	5 ± 2	11 ± 1
β-Alanine	50 ± 13	6 ± 3	31 ± 9
Serine	(*)[c]	(*)	240 ± 13
Total	14,048	773	2,247

[a] Mean ± SE (n = 2). [b] Non detectable. [c] Because of a very close retention time for serine and asparagine, estimation of serine was impossible when a large amount of the amide was present.

Among the organic acids, citrate was the most abundant compound in roots, where it amounted to 49% of the total acidic fraction (Table I). In cytosol, citrate and malate accounted for 35 and 29%, respectively, of the acidic fraction. Malonate and succinate were the other major compounds, and, including citrate and malate, the total of these four acids accounted for 97% of the total organic acid pool in roots and 90% in cytosol. In bacteroids, citrate was much less abundant and represented only 18% of the acidic fraction, whereas malonate and malate were the preponderant compounds. High malonate and malate concentrations have also been reported in bacteroids and cytosol in *Bradyrhizobium japonicum* nodules (21, 23), while the preponderance of succinate has been noticed in white clover nodules (3). Unlike in soybean nodules (21), *trans*-aconitate was not detected in alfalfa nodules or roots. However, lactate appeared significantly in alfalfa, particularly in bacteroids (8% of the acidic fraction). This organic acid was not present in white clover nodules (3) or in soybean nodules (21) but was recently reported to be present in *Vicia faba* roots (29). Finally, fumarate and benzoate were also quantified as minor compounds in alfalfa nodules.

Carbohydrate analysis (Table I) showed a very high sucrose concentration in alfalfa roots (70% of the total carbohydrates) and also in cytosol and bacteroids (53%); it represents the initial supply of reduced carbon entering the nodule from the phloem of the host plant. As in nodules from white clover (3) or in the cytosol of *B. japonicum* nodules (21), sucrose was one of the most abundant sugars. Pinitol was the second major carbohydrate, but its concentration was much lower than that of sucrose: 14% in cytosol and 13% in roots. Substantial quantities of glucose and ononitol were also found, particularly in cytosol and bacteroids. This cyclitol has been characterized as the preponderant carbohydrate extracted from pea nodules (17). *Chiro*-inositol and *myo*-inositol were minor compounds in alfalfa nodules. Each of these metabolites had a low concentration in cytosol and bacteroids (4 to 6% of total carbohydrates), while they are clearly the most abundant water-soluble form of carbon in soybean bacteroids (21). The concentration of trehalose in roots and nodules of alfalfa was extremely low. This disaccharide is synthesized in bacteroids of soybean nodules (20), where its proportional concentration (bacteroid concentration as percentage of cytosol + bacteroid) reaches 66%, but it represented less than 8% in alfalfa bacteroids. However, this low concen-

tration could be related to the relatively young nodule age (34-d-old), because it has been shown that bacteroid forms of *B. japonicum* retained higher proportions of trehalose with increasing nodule age up to 65 d (20).

The amino acid concentration in alfalfa was much higher in nodules than in roots (Table I) as previously shown (25). In the cytosol, asparagine was the most abundant compound (50% of the total amino acids), as expected in an amide-exporting legume. Recently, both high rates of formation of asparagine *in vivo* and high activity of isolated asparagine synthetase *in vitro* have been reported in alfalfa nodules. This enzyme required aspartate, glutamine, ATP, and Mg^{2+}, but ammonium could replace glutamine with a 60% decrease in the activity (26). Results obtained for the other major compounds agreed well with the data of Ta *et al.* (24), who found, in decreasing order, glutamate, γ-aminobutyrate, alanine, proline, and aspartate. The total amino acid concentration in cytosol (14.0 μmol/g fresh weight of nodule) was similar to that reported in soybean nodule cytosol (21) but was four times lower than that found previously in alfalfa nodule cytosol (24). This discrepancy was not surprising, because different cultivars of alfalfa and different strains of *R. meliloti* were used. In addition, our analyses were conducted on nodules collected 34 d after inoculation instead of nodules from 6-week-old plants. In bacteroids, major amino acids were the same as in cytosol. Because the analytical method used here (HPLC) is much more sensitive than the procedure using an amino acid analyzer, we were able to quantify many more amino acids than Ta *et al.* (24). Among these minor compounds, valine, glycine, leucine, isoleucine, and phenylalanine were present in the highest concentrations. Aspartate, the least abundant, accounted for only 0.3% of the total amino acids. In alfalfa roots, glutamate and γ-aminobutyrate are preponderant and represented 25 and 18% of the total amino acid fraction, respectively. Asparagine accounted only for 13%, whereas glutamine reached 6%, a value six times higher than in cytosol or bacteroids.

Effects of Salt Stress on Metabolite Concentrations in Alfalfa Nodules and Roots

Three weeks after inoculation, alfalfa plants were subjected to 0.1 or 0.15 M sodium chloride treatment for 2 weeks. This salt stress had a relatively moderate consequence on the relationship between fresh and dry weight, as well as the correspondence between fresh weight and protein content of nodules and roots (variations never higher than 23%). When a higher salt concentration (0.2 M NaCl) or a longer time application of the 0.15 M NaCl treatment were applied, plant growth and N_2 fixation activity (C_2H_2 reduction) were almost nil. Under the conditions used, the salt treatment (0.15 M NaCl, 2 weeks) decreased nodule number by nearly 50%, and slightly reduced nodule size. Similarly, the N_2 fixation activity (22.1 ± 1.0 μmol $C_2H_4h^{-1}$ g^{-1} fresh weight of nodule, in control plants) was reduced to 30% of the control value.

Salt Stress Effects on Organic Acid Levels

Salt stress strongly decreased the total organic acid concentrations in both nodules and roots of alfalfa plants (Fig. 1). In cytosol, bacteroids, and roots, they were lowered by 43, 49, and 40%, respectively, when 0.15 M NaCl was applied. The concentration of every organic acid was affected, more or less, in the same way, with one important exception in the case of lactate. In cytosol and roots, the lactate concentration increased 11 and 27%, respectively, after a 0.15 M NaCl stress. In bacteroids, this increase was more substantial (94%), and lactate became the most abundant organic acid.

Little is known about organic acid changes induced in legumes subjected to salt or water stress, and these changes are variable between species. Citrate, malate, and malonate, for example, decreased in leaves of various tropical legumes such as *Cicer arietinum* or *Cajanus cajan,* whereas malate and citrate increased in *Glycine max* subjected to water stress (5). An increase in citrate, malate, and lactate and a decrease in glycollate and glycerate have also been observed in *V. faba* roots after withholding of water (29). In alfalfa roots and nodules subjected to salt stress, organic acids did not contribute to the restoration of the osmotic potential and osmotic adjustment, but lactate should be given specific attention, because its concentration was doubled after salt stress. Occurrence of lactate dehydrogenase has been reported in both roots and nodules of *Pisum sativum* (18), and this enzyme is particularly active in the cytosol of stem nodules of *Sesbania rostrata,* in which lactate acts as an energy-yielding substrate for bacteroids (27). Because this organic acid is preponderant in bacteroids from salt-stressed alfalfa, it would be of interest to determine its specific role, *i.e.,* its contribution to the osmotic adjustment or/and involvement in supporting C_2H_2 reduction. In preliminary experiments, intracellular aqueous volumes of bacteroids (6) were measured to estimate the lactate concentration, which increased 2.6-fold during the salt treatment (0.15 M NaCl) but did not exceed 5 mM. Thus, lactate seems to contribute only partly to the osmotic adjustment of the bacteroid.

Salt Stress Effects on Amino Acid Levels

Unlike the response of organic acid concentrations, salt stress did enhance the total amino acid concentration in nodules and roots of alfalfa, resulting in a 3.2-, 2.1-, and 1.6-fold increase in cytosol, bacteroids, and roots, respectively, in the presence of 0.1 M NaCl (Fig. 2). When 0.15 M NaCl was supplied, total amino acid concentrations decreased slightly in cytosol and bacteroids but still increased (11%) in roots. Similar responses have often been reported for other legumes subjected to drought, including *V. faba* (28) and *G. max* (5).

Proline showed the largest proportional increase, followed by asparagine. In the presence of 0.15 M NaCl, the concentration of the amino acid was enhanced 11.3-, 12.8-, and 8.0-fold in roots, cytosol, and bacteroids, respectively. Thus, proline, which accounted for less than 3% in the absence of salt, reached at least 12% of the total amino acids in salt stressed plants. Marked increase (10-fold or more) in free proline occurs in many plants during moderate or severe water or salt stress; this accumulation, mainly as a result of increased proline biosynthesis, is usually the most outstanding change among the free amino acids (9). In soybean nodules, proline has been postulated to play a role in ureide metabolism, in transferring redox potential from the cytosol to the

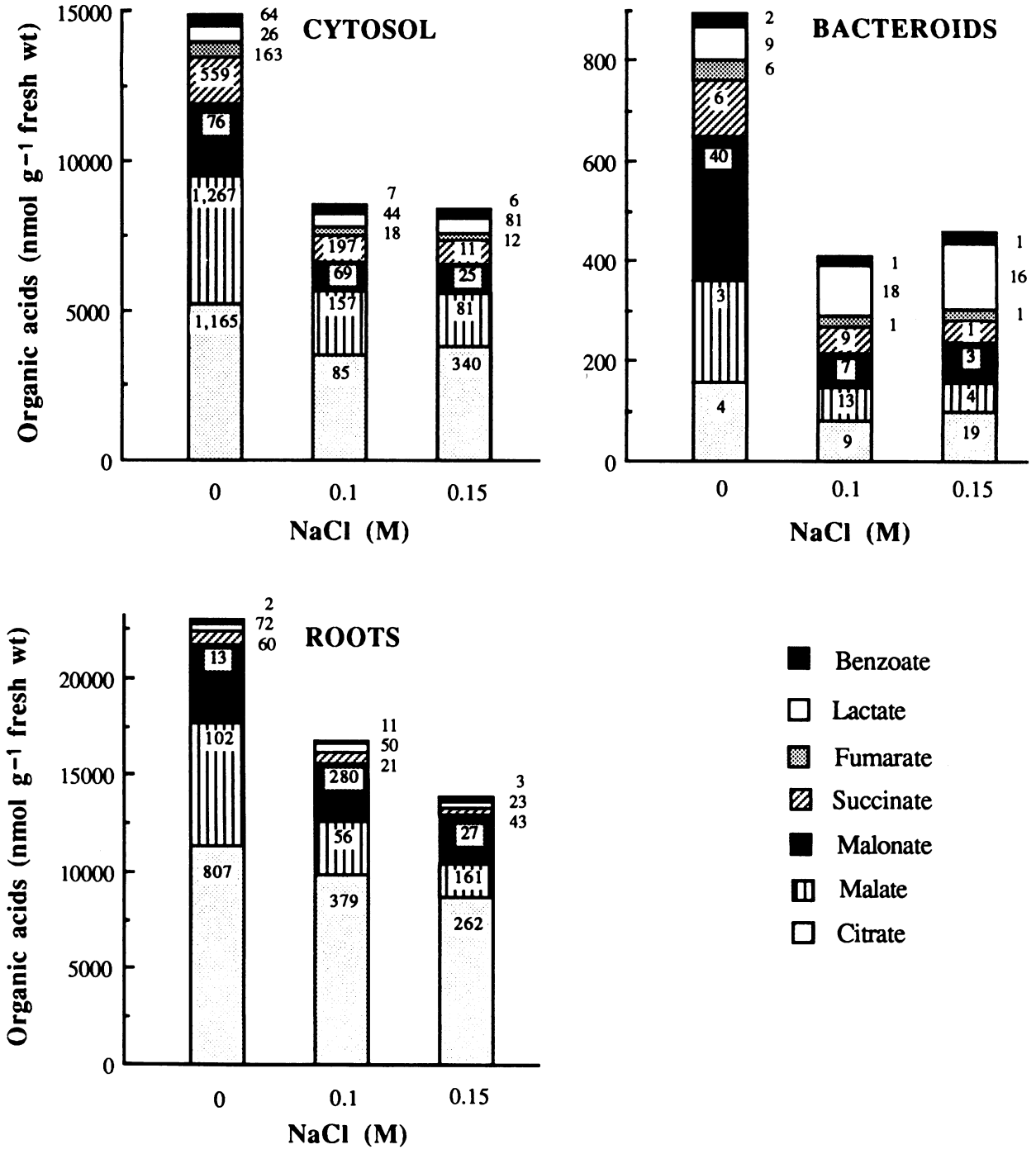

Figure 1. Effect of salt stress on the concentration of organic acids in cytosol, bacteroids, and roots of alfalfa plants. Values are the arithmetic mean of two samples. Numbers indicated in or beside diagrams represent ± SE for each block. The plants were subjected to salt stress (0.1 or 0.15 M NaCl) during 2 weeks. Note that the units on the vertical axis are very different for cytosol, bacteroids, and roots.

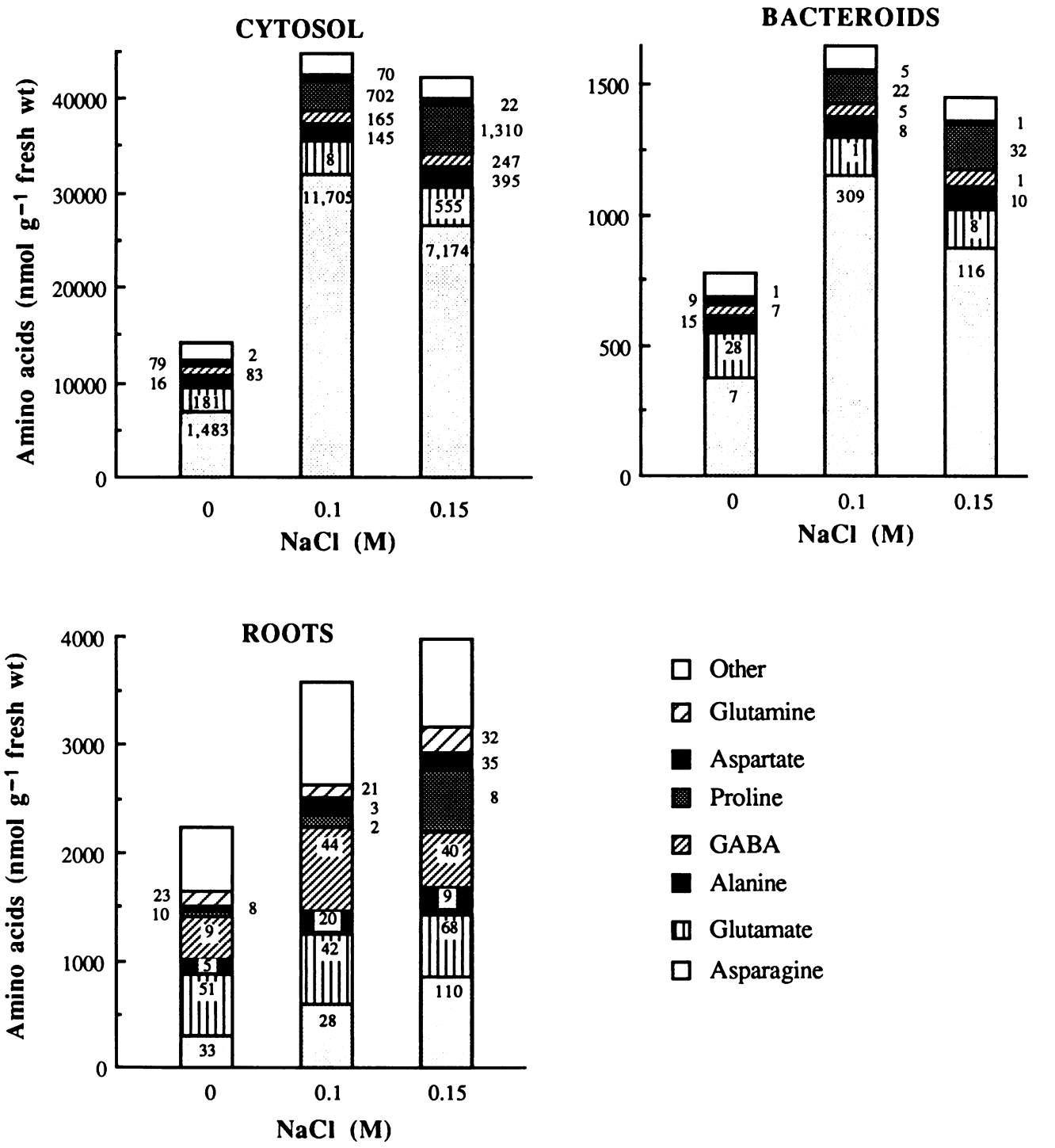

Figure 2. Effect of salt stress on the concentration of amino acids in cytosol, bacteroids, and roots of alfalfa plants. Details are as for Figure 1.

Table II. *Effect of Salt Stress on Carbohydrate Concentration in Cytosol, Bacteroids, and Roots of Alfalfa Plants*

Carbohydrates	Cytosol NaCl (M)			Bacteroids NaCl (M)			Roots NaCl (M)		
	0	0.1	0.15	0	0.1	0.15	0	0.1	0.15
	nmol g^{-1} fresh wt of nodules[a]						*nmol g^{-1} fresh wt*[a]		
Sucrose	15,525	15,179	24,761	750	481	764	11,874	9,229	22,398
	[1,551]	[2,739]	[534]	[130]	[127]	[111]	[101]	[847]	[1,015]
D-Pinitol	4,117	17,351	22,348	216	581	729	2,146	5,889	4,915
	[45]	[276]	[3,763]	[57]	[35]	[57]	[141]	[3]	[153]
Glucose	3,262	3,825	4,199	128	121	160	768	905	1,366
	[499]	[424]	[55]	[35]	[23]	[6]	[36]	[143]	[163]
Ononitol	2,436	5,346	5,874	118	167	184	744	1,421	1,088
	[277]	[307]	[87]	[20]	[19]	[20]	[6]	[146]	[24]
D-*chiro*-Inositol	1,473	553	604	92	27	28	ND[b]	ND	ND
	[594]	[95]	[13]	[52]	[5]	[2]			
myo-Inositol	1,095	1,191	1,444	68	53	66	674	556	1,041
	[137]	[128]	[132]	[22]	[7]	[10]	[6]	[4]	[7]
Fructose	519	566	837	27	43	62	725	795	839
	[97]	[54]	[29]	[2]	[6]	[10]	[138]	[37]	[187]
Maltose	177	303	352	18	37	54	64	110	293
	[42]	[44]	[1]	[3]	[13]	[3]	[12]	[33]	[8]
α-α-Trehalose	101	153	186	8	21	35	83	126	288
	[36]	[32]	[43]	[1]	[2]	[6]	[5]	[23]	[19]
Total	28,705	44,467	60,605	1,425	1,531	2,082	17,078	19,031	32,228

[a] Mean (n = 2); values in brackets are ± SE. [b] Not detectable.

bacteroids, and in serving as a carbon and nitrogen source for these bacteroids (11). Indeed, soybean nodules contain a high level of Δ^1-pyrroline-5-carboxylate reductase activity, which is localized in the cytosol (11) and catalyzes the terminal reaction in proline biosynthesis from glutamate. Moreover, it was shown recently that exposure of soybean seedlings to 0.2 or 0.4 M NaCl caused a four- to sixfold increase in the level of Δ^1-pyrroline-5-carboxylate reductase mRNA in roots (4), suggesting that this gene may be osmoregulated and, thus, reflecting an osmoregulatory function for proline synthesis in nodule tissue.

The proportional increase in asparagine concentration was lower than that of proline concentration, between 2.4-fold in bacteroids and 3.8-fold in cytosol under 0.15 M NaCl stress. In nodules, asparagine remained by far the major compound (at least 60% of the total amino acid pool), whereas it represented only 22% in roots. In bacteroids from salt-stressed plants (0.15 M NaCl), the amide concentration reached 30 mM, which represents a significant contribution to the osmotic adjustment. Marked increase in asparagine content has already been reported in stems and roots of *V. faba* (28) grown under drought conditions, but, to our knowledge, not in the cytosol or bacteroids. Surprisingly, a sixfold increase in asparagine concentration in *B. japonicum* bacteroids was also observed after an 8-d treatment with 15 mM nitrate. However, a specific effect of nitrate on asparagine accumulation was not clearly indicated (22). In alfalfa nodules, asparagine is synthesized by amidation of aspartate, where both glutamine and NH_3 act as N donors (26). It will be of interest to determine the effect of salt stress on asparagine synthetase activity from alfalfa nodules. However, the salt effect may also relate to effects on breakdown or export of asparagine from the nodule.

The salt treatment had a very limited effect on the concentration of other amino acids. They all increased slightly (average 1.5-fold), except the glutamate concentration, which stayed constant in roots and decreased slightly (16%) in bacteroids.

Salt Stress Effects on Carbohydrate Concentrations

In cytosol, bacteroids, and roots of alfalfa subjected to the salt treatment (0.15 M), total carbohydrates increased 2.1-, 1.5-, and 1.9-fold, respectively, compared to the control (Table II). Unlike the total amino acid pool, this increase appeared mainly when the salt concentration was raised from 0.1 to 0.15 M NaCl.

Except *chiro*-inositol concentration, which decreased 2.4- and 3.3-fold in cytosol and bacteroids, respectively, and *myo*-inositol as well as sucrose concentrations, which remained constant in bacteroids, all other carbohydrate concentrations increased substantially. Several points should be emphasized. First, sucrose, which moderately increased in roots and cytosol (1.9- and 1.6-fold, respectively), remained the predominant carbohydrate, and accounted at least for 37% of the carbohydrate pool in nodules and up to 70% in roots. Second, pinitol concentration strongly increased in cytosol (5.4-fold) and bacteroids (3.4-fold) and comprised 35 to 37% of the carbohydrate pool after a 0.15 M NaCl stress. In roots, increase in pinitol was less pronounced (2.3-fold). Thus, salt stress only slightly modified the pinitol/sucrose ratio in roots (from 0.18–0.22), while it highly favored its increase in cytosol (from 0.27–0.90) and also in bacteroids (from 0.29–0.95). Third, among less abundant carbohydrates, maltose and trehalose concentrations were significantly enhanced by the salt treatment, especially in roots (4.6- and 3.5-fold, respectively) and

bacteroids (3.0- and 4.4-fold, respectively). However, these sugars remained minor compounds, less than 2.5% of the carbohydrate pool. Fourth, the concentration of other solutes, like glucose, ononitol, and fructose, increased more or less in proportion to the total carbohydrate pool.

Although a common feature of plants, including legumes, is the accumulation of low mol wt carbohydrates under salt stress condition or drought (5, 15), the effects of salt stress on carbohydrate fluctuations had not been investigated in cytosol and bacteroids before. From our results, it is tempting to suggest that pinitol functions as a compatible solute in cytosol and bacteroids from alfalfa nodules, where it could make a very significant contribution to the osmotic adjustment. As an example, in bacteroids, the concentration of pinitol increased 4.5-fold under salt stress (0.15 M NaCl), and reached 25 mM, a value almost similar to that obtained for asparagine. Pinitol was also shown to accumulate after the application of NaCl in the salt-tolerant legume *S. bispinosa* (7). In addition, data obtained with soybean and white clover have suggested that pinitol is not rapidly metabolized in these plants (19). Similarly, recent results for *Mesembryanthemum crystallinum* indicate a metabolically inert role for this cyclitol (15). Corroboration of this hypothesis will give additional importance to the role of pinitol as an osmoregulatory compound. An equivalent role for most other carbohydrates seems unlikely, because their proportional concentration tended to decrease or remain constant with salt stress. Among the few exceptions, trehalose increased in bacteroids. This disaccharide, known to be synthesized in bacteroids of *B. japonicum* (20), has been shown to serve as an osmoprotective compound in *Escherichia coli* (12). However, in alfalfa bacteroids the concentration of trehalose was too low to contribute efficiently in osmoregulation.

In summary, it can be concluded that salt stress strongly decreased the organic acid pool in nodules of alfalfa, but it should be emphasized that lactate concentration increased significantly in bacteroids, where it became the preponderant organic acid, and might contribute partially to the osmotic adjustment. Among amino acids, proline showed the largest increase under salt stress and was accumulated in cytosol and bacteroids. As reported in roots, stems, and leaves of other plants, proline accumulation in nodules may represent an efficient osmoregulatory mechanism. The parallel salt stress-induced high concentration of asparagine in nodule tissues might also reflect an osmoregulatory function for this amide. We can also tentatively assume that pinitol serves as a compatible solute, and its large accumulation in nodules could contribute to the tolerance to salt stress.

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